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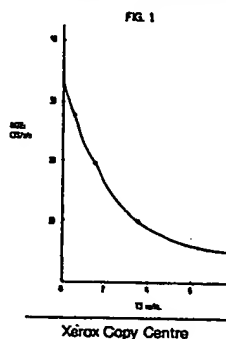
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⑤④ **Thyroid hormone assay and reagent system employing furosemide.**

⑤⑦ A method for measuring the concentration of a thyroid hormone selected from the group consisting of thyroxine (T_4) and triiodothyronine (T_3) in a sample comprising the steps of treating a sample with furosemide to displace T_3 and T_4 from binding proteins and measuring the amount of T_3 and T_4 displaced. A thyroid hormone reagent system is also disclosed to measure the concentration of thyroxine (T_4) and triiodothyronine (T_3) in a sample comprising furosemide in an amount sufficient to substantially displace all T_3 and T_4 bound to binding proteins in a sample.



EP 0 312 897 A1

Thyroid Hormone Assay and Reagent System Employing Furosemide

The present invention is directed toward a method for measuring thyroid hormones such as thyroxine (T_4) and triiodothyronine (T_3) in biological samples. The method comprises the use of furosemide (4-chloro-N-(2-furylmethyl)-5-sulfamoylanthranilic acid) or analogs thereof to displace the thyroid hormones from the binding proteins such that the thyroid hormones can be measured with suitable assay reagents. Furosemide has the advantage of not inhibiting the binding of the thyroid hormones or tracers to the antibody employed in the assay because it has very low crossreactivity with antibody.

The measurement of T_3 and T_4 concentrations in biological samples such as plasma and serum is very valuable in the diagnosis and treatment of various diseases and physiological conditions. In normal human serum, about 99.97% of T_4 and 99.7% of T_3 present, is bound to proteins such as thyroxine-binding globulin (TBG), prealbumin (TBPA) and albumin; the remaining 0.03% of T_4 and 0.3% of T_3 is present as unbound (dialyzable or free) hormones. The serum concentration of thyroid hormones, along with other factors such as thyroidal secretion and degradation, is also dependent on serum concentration of TBG; other binding proteins in serum are relatively less important since their binding affinity for thyroid hormones is far less than that of TBG. The concentration of TBG is elevated in conditions such as pregnancy, estrogen treatment or a genetic abnormality; serum total T_4 and T_3 is increased in these situations. Conversely, serum TBG concentration is decreased during treatment with androgens or due to a genetic deficiency of TBG. This is associated with decreased concentrations of thyroid hormones. However, in either of the above-mentioned situations of altered TBG concentration, the patient can be euthyroid and concentrations of free T_4 and T_3 and that of total T_4 and T_3 corrected for TBG abnormality are within the range of normal subjects.

U.S. Patent 3,911,096 discloses a radioimmunoassay method for determining T_3 or T_4 concentrations in human serum samples. The method incubates serum sample with barbital or other buffers to displace T_3 or T_4 with a "blocking agent", radioactive labeled T_3 or T_4 and antibody specific for T_3 or T_4 . The amount of bound radioactive T_3 or T_4 is then measured to determine hormone concentration against a standardized curve. The "blocking agents" can include 8-anilino-1-naphthalene-sulfonic acid (ANS), 3-(4-anilino-1-naphthylazo)-2,7-naphthalene disulfonic acid (ANNDs), 2,4,6-trinitro benzene sulfonic acid (TNBS), naphthalene sulfonic acid, Thimerosal, 5,5-diphenyl 2-thiohydantoin, Doxepin HCl, Diazepam, sodium salicylate, prochlorperazine, halofenate (MK-185) Merck, Sharpe and Dohme.

More recent methods do not use barbital or buffers but instead only employ the so called "blocking agents" as described in U.S. Patent 3,911,096 such as ANS. A disadvantage of ANS is that it is light sensitive which can interfere with absorption measurements.

Older methods used solvent extraction, enzymic digestion of the sample or other agents to extract the T_3 or T_4 hormone. These methods have proved to be cumbersome and the treatment steps have interfered with the accurate determination of the thyroid hormone concentrations. Therefore an improved method for the determination of T_3 and T_4 hormones is desirable.

Summary of the Invention

The present invention is directed toward a method for measuring the concentration of a thyroid hormone selected from the group consisting of thyroxine (T_4) and triiodothyronine (T_3) in a sample comprising the treatment of a sample with furosemide to displace T_3 and T_4 from binding proteins and measuring the amount of T_3 and T_4 displaced. The amount of T_3 and T_4 displaced is measured using any of a variety of known thyroid hormone assay reagents and methods. Preferably, the furosemide replaces any other displacing agent or "popper" and displacing steps formerly employed in a conventional reagent system or method.

The amount of furosemide used is calculated to displace substantially all T_3 and T_4 as a function of sample size. The method of detection can be fluorescence, absorption, fluorescence polarization, chemiluminescence or radioactive means.

In another aspect, the present invention is directed toward a reagent system designed to measure the concentration of a thyroid hormone selected from the group consisting of thyroxine (T_4) and triiodothyronine (T_3) in a sample comprising furosemide in an amount sufficient to substantially displace all T_3 and T_4 bound to binding proteins in a sample.

The remainder of the reagent system can be any of a variety of thyroid reagent systems. Preferably,

the furosemide replaces all the conventional displacing agent or "popper" or displacing step generally employed in such a thyroid reagent system. The reagent systems and methodologies can comprise fluorescent enzyme-immunoassay, fluorescent polarization immunoassay, chemiluminescence immunoassay, radioimmunoassay and colorimetric enzyme-immunoassay to name a few.

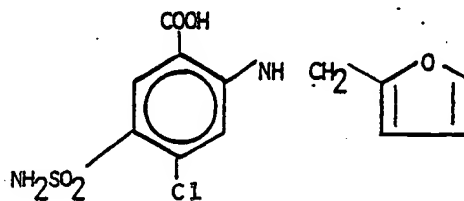
Detailed Description of the Invention

A method of determining thyroxine (T_4) and triiodothyronine (T_3) in biological samples such as whole blood, serum and plasma is described. The method uses furosemide as a displacing agent in immunoassays for the thyroid hormones. It displaces the hormones from their protein binders such as thyroxine-binding globulin (TBG), albumin, and prealbumin such that the direct measurement of these hormones in serum or plasma can be made with an appropriate antibody.

Furosemide is particularly advantageous as a displacing agent because it does not interfere with the binding of the analyte T_3 or T_4 or the tracer (i.e., T_3 or T_4 derivatives such as enzyme, enzyme inhibitors, fluorescent, or radioactive labels) to the antibody. This is an advantage over other classical displacing agents such as 8-anilino-1-naphthalene-sulfonic acid (ANS) or salicylate. Further, furosemide retains its solubility in the assay reagent system which improves its handling characteristics.

Furosemide is directly added to the sample with the reagent used to identify the free thyroid hormone. It can be employed in any thyroid reagent system such as those designed for fluorescence, absorption, chemiluminescence or radioimmunoassays. Thus, furosemide is a convenient additive for a thyroid assay reagent system as a displacing agent or "popper". More preferably, furosemide can be employed to completely eliminate any other displacing agent or hormone displacing steps formerly employed in an otherwise conventional thyroid assay reagent system and method. The quantity of furosemide employed is dependent on the amount required to displace the hormone from the binding protein(s) under the conditions of the particular assay. This can easily be determined by those skilled in the use of thyroid hormone assays as a function of available sample to be tested.

Furosemide (4-chloro-N-(2-furylmethyl)-5-sulfamoylanthranilic acid) can be represented by the structural formula as follows:



Analogous or derivatives of furosemide which can produce similar results as a displacing agent for a thyroid reagent system and are therefore contemplated to be within the scope of this invention.

The method of the subject invention uses furosemide as the displacing agent for the thyroid hormones in any of a variety of thyroid reagent system. The furosemide is added to the sample in an amount sufficient to displace substantially all, preferably all, the available bound thyroid hormone (T_3 and T_4). The sample can be either treated with furosemide before adding the reagent system chosen to quantitate the released thyroid hormone or it can be added simultaneously with the reagent system.

The furosemide displacing agent can be employed in any of a variety of thyroid reagent systems to quantitate the thyroid hormone displaced by either competitive or noncompetitive assay techniques. Also the furosemide supplemented reagent system can be employed with any of a variety of detection methods such as fluorescence, absorption, fluorescence polarization, chemiluminescence, or radioactivity. Typical labels used in thyroid hormone assays and compatible with furosemide can include fluorescent enzyme labels, chemiluminescent labels, colorimetric labels, luminous labeled materials, radioactive labels and metal or nonmetal labels.

The following examples illustrate the use of furosemide as a displacing agent in the measurement of thyroid hormones.

EXAMPLE I

This example uses furosemide as a displacing agent in the measurement of thyroid hormones and illustrates the making of a standard curve, to allow accurate correlation of unknown T_4 concentrations with a standard curve.

The reagents employed in this enzyme inhibitor immunoassay were acetylcholinesterase, furosemide and a T_4 -binding antiserum or T_4 antibody obtained from a sheep immunized with thyroxine conjugated to bovine thyroglobulin. The T_4 antibody was used in a final dilution of 1:60,000. The steps of the enzyme inhibitor immunoassay procedure were performed as described below.

A first aqueous reagent consisted of 16.6 microliters per liter (uL/L) of sheep antithyroxine antiserum, 34 units per liter (U/L) of acetylcholinesterase and 0.7 millimoles per liter (mM/L) of furosemide in a Hepes buffer with protein stabilizer (0.6 moles per liter lithium sulfate).

A second aqueous reagent consisted of 8 nanomoles per liter (nM/L) of thyroxine-inhibitor conjugate, 2 mM/L of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and 3.85 mM/L of S-acetyl-B-(methylthio)choline iodide in a citrate buffer.

The first reagent solution was added to 3.6 microliters of plasma in an amount of 168 microliters and mixed. This mixture was allowed to incubate for approximately five minutes. Next, 85 microliters of the second reagent solution was added and absorbance readings taken at 415 and 500 nanometer wavelengths at defined time intervals.

In this thyroid hormone assay, sample thyroxine competes with an enzyme inhibitor (T_4 -conjugate), which is structurally similar to thyroxine, for binding sites on a specific antibody. The amount of free conjugate is determined by measuring the rate at which enzyme activity is lost. T_4 -conjugate and specimen thyroxine are bound in proportion to their respective concentrations in solution. Furosemide is used to release thyroxine from serum binding proteins and prevent subsequent binding.

This test utilized 5 calibrators with nominal amounts of thyroxine at the following concentrations 0, 3, 6, 12 and 24 micrograms per deciliter (ug/dL). The time constant for disappearance of enzyme activity is fit to a 4 parameter logistic curve to generate a calibration curve. The concentration range of the thyroxine calibrators (0-24 ug/dL) is adequate to accurately determine the thyroxine concentration in most patient samples encountered. When samples containing thyroxine in concentrations greater than the highest calibrator (24 ug/dL) are encountered they can be accurately determined by diluting the sample with the calibrator and reassaying. The sample thyroxine value is calculated by multiplying the result from the diluted sample by the dilution factor.

EXAMPLE II

This example uses furosemide as a displacing agent in the measurement of thyroid hormones in an enzyme-immunoassay. The reagents employed were as follows:

T_3 binding antiserum bound to polystyrene particles obtained from a goat immunized with a covalent conjugate of T_3 and bovine thyroglobulin. The antibody coated particles are diluted such that the proper dose response is obtained in the assay. The furosemide is present in this reagent at a concentration of 0.0125% (weight/volume).

A T_3 -alkaline phosphatase conjugate was prepared and diluted in buffer containing protein stabilizers.

Reagent grade Na-L- T_3 was added gravimetrically to T_3 free human serum to a final concentration of 0, 0.5, 1, 2, 4 and 8 nanograms per milliliter (ng/ml). These materials served as calibrators.

The substrate consisted of an aqueous buffered solution of 4-methylumbelliferoyl phosphate. The assay diluent consisted of 50 millimolar of Tris, 0.3 molar NaCl, pH 7.5.

The following steps were carried out for this assay.

Fifty microliters (uL) of calibrator or sample was added to a polystyrene reaction well. To this was added 50 uL of particle reagent followed by 150 uL of assay diluent. This mixture was mixed and incubated for approximately ten minutes at 34.5° C. The furosemide in the particle reagent effectively displaced the T_3 from binding protein.

A 175 uL volume of the mixture was transferred to a glass fiber matrix and washed with two 75 uL aliquots of assay diluent. The particles to which released T_3 was bound were irreversibly bound to the matrix.

The T_3 alkaline phosphate conjugate reagent was added to the matrix and after a four minute incubation unreacted conjugate was washed from the matrix with two 75 uL aliquots of assay diluent.

Substrate was then added to the matrix and the rate of formation of product, methyl umbelliferone, was

determined by fluorescent emission.

Figure 1 shows a typical standard curve plotted as rate in counts per second per second (CTS/s/s) versus the amount of bound T_3 measured in nanograms per milliliter (ng/ml). The fifty percent intercept is 2.0 ng/ml and the sensitivity of the assay is measured by its ability to distinguish a sample from zero with 95% confidence in 0.13 ng/ml. The plot indicates the excellent results obtained with the thyroid reagent system containing furosemide.

Table 1 illustrates T_3 values obtained in the assay of Example II (EIA) compared to a typical radioimmunoassay (RIA) which utilizes 8-anilino-1-naphthalene-sulfonic acid (ANS) as a deblocking agent.

TABLE I

| Sample | EIA with furosemide | RIA with ANS |
|--------------|---------------------|-----------------|
| low T_3 | 0.42 ± 0.20 | 0.51 ± 0.18 |
| normal T_3 | 1.53 ± 0.25 | 1.60 ± 0.31 |
| hyperthyroid | 4.19 ± 1.36 | 4.20 ± 1.24 |

The results indicate that using furosemide as a displacing agent yields valid sample values in that the assay is able to distinguish low, normal and high T_3 samples, i.e., it is as effective as classical methodologies in displacing T_3 from its binding proteins.

Claims

1. A method for measuring the concentration of a thyroid hormone selected from the group consisting of thyroxine (T_4) and triiodothyronine (T_3) in a sample comprising:

- a) treating a sample with furosemide to displace T_3 and T_4 from binding proteins;
- b) measuring amount of T_3 and T_4 displaced.

2. The method of Claim 1 wherein the amount of furosemide is calculated to displace substantially all T_3 and T_4 as a function of sample size.

3. The method of Claim 2 wherein said step (b) is conducted by fluorescence, absorption, fluorescence polarization, chemiluminescence or radioactive means.

4. The method of Claim 1 wherein said sample is a plasma or serum sample.

5. A reagent system designed to measure the concentration of a thyroid hormone selected from the group consisting of thyroxine (T_4) and triiodothyronine (T_3) in a sample comprising: - furosemide in an amount sufficient to substantially displace all T_3 and T_4 bound to binding proteins in a sample.

6. The reagent system of Claim 5 which is fluorescent enzyme-immunoassay.

7. The reagent system of Claim 5 which is a chemiluminescence immunoassay.

8. The reagent system of Claim 5 which is a radioimmunoassay.

9. The reagent system of Claim 5 which is a colorimetric enzyme-immunoassay.



| DOCUMENTS CONSIDERED TO BE RELEVANT | | | |
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| The present search report has been drawn up for all claims | | | |
| Place of search THE HAGUE | | Date of completion of the search 01-02-1989 | Examiner GRIFFITH G. |
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| Place of search THE HAGUE | | Date of completion of the search 01-02-1989 | Examiner GRIFFITH G. |
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